Rac-dependent cyclin D1 gene expression regulated by cadherin- and integrin-mediated adhesion

Alaina K. Fournier\textsuperscript{1}, Latoya E. Campbell\textsuperscript{1}, Paola Castagnino\textsuperscript{1}, Wendy F. Liu\textsuperscript{2}, Betty M. Chung\textsuperscript{1}, Valerie M. Weaver\textsuperscript{3,4}, Christopher S. Chen\textsuperscript{2} and Richard K. Assoian\textsuperscript{1,4}

 Departments of \textsuperscript{1}Pharmacology, \textsuperscript{2}Bioengineering, and \textsuperscript{3}Pathology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6084, USA
\textsuperscript{*}Author for correspondence (e-mail: rka@pharm.med.upenn.edu)

Accepted 25 October 2007
Journal of Cell Science 121, 226-233 Published by The Company of Biologists 2008
doi:10.1242/jcs.017012

Summary
Integrin-mediated adhesion to substratum is required for cyclin D1 induction in mesenchymal cells, but we show here that the induction of cyclin D1 persists despite blockade of ECM-integrin signaling in MCF10A mammary epithelial cells. E-cadherin-mediated cell-cell adhesion also supports cyclin D1 induction in these cells, and the combined inhibition of both E-cadherin and integrin adhesion is required to prevent the expression of cyclin D1 mRNA and protein. Our previous studies described a pro-proliferative effect of E-cadherin in MCF10A cells, mediated by Rac, and we now show that Rac is required for cyclin D1 mRNA induction by both E-cadherin and integrin engagement. The levels of p21Cip1 and p27Kip1, Cdk inhibitors that are also targets of integrin signaling, are not affected by E-cadherin-mediated cell-cell adhesion. Finally, we show that the increased expression of cyclin D1 mRNA associated with E-cadherin-dependent cell-cell adhesion is causally linked to an increased entry into S phase. Our results identify Rac signaling to cyclin D1 as a crucial pro-proliferative effect of E-cadherin-mediated cell-cell adhesion.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/2/226/DC1

Key words: G1 phase, Cell cycle, Proliferation

Introduction
Progression through the cell cycle is controlled by the activity of cyclin-dependent kinases (Cdks) (Sherr, 1994; Sherr and Roberts, 1999). Cyclin D1 is the major D type cyclin in many cell types and is usually the first cyclin to be induced when cells enter G1 phase from quiescence (G0). Once expressed, cyclin D1 binds to Cdk4 or Cdk6 to form an active holoenzyme that phosphorylates the retinoblastoma 1 (Rb1) protein. Phosphorylation of Rb1 allows for the dissociation of associated E2Fs which then promote transcription of downstream E2F cell cycle targets including cyclin E and cyclin A. The cyclin-D1–Cdk4/6 complex also promotes G1 phase cell-cycle progression by titrating Cdk inhibitors, p21Cip1 and p27Cip1, and thereby contributing to the activation of cyclin-E–Cdk2 complexes that further phosphorylate Rb1. Tight control of cyclin D1 gene expression is therefore a crucial issue in the regulation of G1-phase progression.

In fibroblasts, the induction of cyclin D1 mRNA requires coordinated signaling by growth factor receptor tyrosine kinases (RTKs) and integrins. For example, in the presence of growth factors, integrin-mediated cell adhesion to the extracellular matrix (ECM) leads to a sustained activation of ERKs (also known as MAPKs) that is required for cyclin D1 gene expression (Welsh et al., 2001; Villanueva et al., 2007). RTKs and integrins also regulate the activation of Rac (RAC1), and integrin signaling additionally controls the coupling of Rac to its downstream targets (del Pozo et al., 2000). Although cyclin D1 is induced downstream of activated Rac (Joyce et al., 1999; Klein et al., 2007; Page et al., 1999), endogenous Rac signaling to cyclin D1 is not readily detected in fibroblasts because the pathway is inhibited by Rho (Welsh et al., 2001).

Epithelial cells have more complex adhesion systems than fibroblasts. In addition to integrin-mediated adhesion to the ECM, epithelial cells rely on adherens junctions for tissue integrity and function, and E-cadherin plays a major role in mediating these adherens junctions in many epithelial cell types. E-cadherin is a transmembrane protein that mediates cell–cell adhesion by calcium-dependent homophilic binding through its extracellular domain (Gumbiner, 1996). β-catenin binding to the cytoplasmic domain of E-cadherin acts as a link to the actin cytoskeleton (Drees et al., 2005; Knudsen et al., 1995; Nieset et al., 1997; Yamada et al., 2005). A current hypothesis suggests that cadherin-mediated binding of β-catenin may affect catenin-dependent transcription of LEF-regulated genes (Gottardi et al., 2001; Sadot et al., 1998). Interestingly, the cyclin D1 gene can be regulated by β-catenin and LEF (Shutman et al., 1999; Tetsu and McCormick, 1999), raising the possibility that the formation of E-cadherin adherens junctions might control the expression of cyclin D1 by sequestering β-catenin. However, E-cadherin can also regulate Rac activity (Nakagawa et al., 2001; Noren et al., 2001; Liu et al., 2006) and therefore has the potential to regulate Rac-dependent induction of cyclin D1.

We recently reported that E-cadherin stimulates Rac-GTP loading and promotes cell proliferation in a Rac-dependent manner in MCF10A cells (Liu et al., 2006). However, the pro-proliferative target(s) of E-cadherin within the G1 phase cyclin-Cdk network remained undefined. We now describe the effects of E-cadherin on the G1-phase cyclins and Cdk inhibitors, link Rac signaling to cyclin D1 mRNA, and assess the relative effects of integrin-mediated cell-substratum adhesion and cadherin-mediated cell-cell adhesion on these events in MCF10A mammary epithelial cells.
Our results reveal clear differences between the adhesion requirements for cyclin D1 gene expression and S-phase entry in mesenchymal and epithelial cells.

**Results**

**Cyclin D1 gene expression persists in MCF10A cells despite loss of cell-substratum adhesion**

Our initial studies examined the induction of cyclin D1 when quiescent MCF10A cells were stimulated with mitogens on dishes coated with collagen (monolayer cultures) or agarose (suspension cultures), the latter being an experimental condition that blocks integrin clustering and downstream signaling. Although cyclin D1 levels were reduced by blocking cell adhesion to the substratum, cyclin D1 was still induced relative to G0 in these suspended epithelial cells (Fig. 1A). Cell adhesion to ECM is thought to control cyclin D1 expression, at least in part, by regulating ERK activity (Assoian and Schwartz, 2001), but we found that cyclin D1 mRNA was induced in suspended MCF10A cells even though ERK activity was absent (Fig. 1B). These results are in striking contrast to our previous studies with human and mouse fibroblasts where blocking integrin-mediated adhesion to the substratum strongly inhibited cyclin D1 induction as well as the activation of ERKs (Bohmer et al., 1996; Zhu et al., 1996; Roovers et al., 1999; Welsh et al., 2001). In addition to integrins, numerous cell-cell adhesion systems including cadherins may be responsible for the formation of spheroids. We therefore analyzed the MCF10A cell aggregates that were collected by attachment to poly-L-lysine-coated coverslips and visualized by phase-contrast microscopy. Bar, 210 μm. (D) Attachment of MCF10A cells to collagen (COLL), vitronectin (VN), fibronectin (FN) or laminin-1 (LM) in the absence and presence of AIIβ2 and/or RGD as described in the Materials and methods. The results are plotted as percentage maximal attachment relative to the positive control (no inhibitors) for each matrix protein. We noticed that MCF10A cells began to form loose aggregates after ~3 hours in suspension and then proceeded to form spheroid-like structures by 9 hours (Fig. 1C, first column). The same effects were seen when the cells were incubated in serum-free medium (Fig. 1C, second column), indicating that serum-derived matrix proteins were not responsible for the aggregation. Nevertheless, we directly inhibited any residual integrin signaling by treating the cells with a β1-integrin-blocking antibody AIIβ2 and the RGD peptide. The individual inhibitors had the largely expected effects on integrin-mediated adhesion, and the combination of both inhibitors abolished attachment to collagen, laminin, vitronectin and fibronectin (Fig. 1D). However, cell aggregation in suspension was not diminished by treatment with the integrin inhibitors (Fig. 1C, third column). Taken together, the results of Fig. 1 indicated that another adhesion system was responsible for the tight cell aggregation in suspended MCF10A cells, and that this system might also be responsible for the ERK-independent expression of cyclin D1.

**E-cadherin regulates cyclin D1 via Rac**

In addition to integrins, numerous cell-cell adhesion systems including cadherins may be responsible for the formation of spheroids. We therefore analyzed the MCF10A cell aggregates that formed in suspension culture for the expression and localization of E-cadherin and β-catenin by immunofluorescence microscopy. Both E-cadherin and β-catenin localized to the cell-cell interface (Fig. 2A). Thus, E-cadherin-mediated cell-cell adhesion was intact in the suspended cell aggregates. Knock-down of E-cadherin with small interfering RNA (siRNA) (Fig. 2B) inhibited the formation of tight spheroids in suspended MCF10A cells (Fig. 2C), and...
largely phenocopied the effect of disrupting cadherin-mediated adhesions by calcium chelation with EGTA (Fig. 2D). These results show that E-cadherin-mediated cell-cell adhesion is responsible, at least in large part, for the formation of spheroids in nonadherent MCF10A and suggest that E-cadherin might be responsible for the integrin-independent expression of cyclin D1.

We transfected MCF10A cells with either control or E-cadherin siRNA and then cultured them in monolayer or suspension. Western blotting for E-cadherin or Y397-phosphorylated FAK confirmed that these treatments were effective inhibitors of E-cadherin and integrin signaling, respectively. Inhibition of E-cadherin signaling in monolayer cultures (Fig. 3A,B; E-cadherin siRNA; Mono) or integrin signaling in suspension cultures (Fig. 3A,B; control siRNA; Susp) partially inhibited the induction of cyclin D1 mRNA and protein, though the magnitude of the effects varied somewhat between experiments. By contrast, the inhibition of both E-cadherin and integrin signaling (knock-down of E-cadherin in suspended MCF10A cells) consistently resulted in a strong inhibition of cyclin D1 induction (Fig. 3A,B; E-cadherin siRNA in Susp). Similar results were obtained with two distinct E-cadherin siRNAs (supplementary material Fig. S1) and a dominant-negative E-cadherin adenovirus lacking the cytoplasmic domain (supplementary material Fig. S2). Interestingly, we did not see E-cadherin-dependent changes in the levels of p21 or p27, the Cdk

Fig. 2. E-cadherin-mediated adhesion in suspended MCF10A cells.
(A) Quiescent MCF10A cells were trypsinized, reseeded on dishes coated with agarose, stimulated with 10% FBS and growth factor cocktail for 9 hours, and analyzed by phase-contrast or epifluorescence microscopy for E-cadherin (E-cad) and β-catenin (β-cat). (B) MCF10A cells were transfected with control or E-cadherin siRNA, rendered quiescent by serum and growth factor starvation, trypsinized, reseeded on collagen-coated dishes, stimulated with 10% FBS and growth factor cocktail for 9 hours, and analyzed by epifluorescence microscopy for E-cadherin, β-catenin and DAPI-stained nuclei. (C) Cells prepared as in B were seeded on agarose-coated dishes with 10% FBS and growth factor cocktail for 9 hours, collected on poly-L-lysine-coated coverslips, and analyzed by phase-contrast microscopy. (D) Quiescent MCF10A cells were trypsinized and pre-incubated in suspension in the absence and presence of 2 mM EGTA (30 minutes at 37°C) in DMEM-F12 with 1 mg/ml heat-inactivated fatty acid-free BSA. The pre-treated cells were directly replated on agarose-coated dishes, and stimulated with 10% FBS and growth factor cocktail for 9 hours. The cells were then collected on poly-L-lysine-coated coverslips and analyzed by phase-contrast microscopy. Bars, 210 μm.

Fig. 3. E-cadherin-mediated cell-cell adhesion and cell-substratum signaling coordinately regulate cyclin D1 expression in MCF10A cells. MCF10A cells were transfected with control or E-cadherin (E-cad) siRNA, rendered quiescent by serum and growth factor starvation, trypsinized, and reseeded in a monolayer (Mono) or in suspension (Susp) on collagen- or agarose-coated dishes, respectively. The cells were stimulated with 10% FBS and growth factor cocktail. (A) Samples were collected at the indicated time points and analyzed by western blotting for cyclin D1, Cdk6, E-cadherin, β-catenin, phosphoY397-FAK and FAK. (B) The experiment in A was repeated with cells seeded in monolayer (on collagen-coated dishes containing glass coverslips) (Liu et al., 2006) or in suspension (on agarose-coated dishes) at 2×10^4 cells/cm². Collected cells were analyzed by QPCR for cyclin D1 mRNA. (C) Cells seeded on collagen-coated dishes and stimulated with 10% FBS and growth factor cocktail were collected and analyzed by western blotting for E-cadherin, p21Cip1, p27Kip1, dually phosphorylated ERK, total ERK and GAPDH (loading control).
E-cadherin regulates cyclin D1 via Rac inhibitors that are often targeted by extracellular signals in G1 phase (Fig. 3C). Moreover, ERK activity was not affected by knock-down of E-cadherin (Fig. 3C), indicating that cadherin induction of cyclin D1 operated through a distinct signaling pathway.

We conclude that cyclin D1 is the major G1 phase pro-proliferative target of E-cadherin in MCF10A cells and that integrins and E-cadherins are each sufficient to induce cyclin D1. The combined inhibition of both adhesion systems results in the near complete loss of cyclin D1 mRNA and protein expression. Thus, E-cadherin- and integrin-mediated adhesion account for essentially all the adhesion dependency of cyclin D1 mRNA expression in MCF10A cells. Additionally, we found that knock-down of E-cadherin did not affect cyclin D1 protein stability in suspended MCF10A cells (Fig. 4A), nor could E-cadherin knock-down reduce the dose-dependent expression of an ectopically expressed cyclin D1 coding domain (Fig. 4B). Thus, the change in steady-state cyclin D1 protein levels associated with knock-down of E-cadherin largely reflects the stimulatory effect of E-cadherin on cyclin D1 mRNA.

E-cadherin-mediated cyclin D1 gene expression in MCF10A cells is dependent on Rac

Others have reported that E-cadherin-mediated adhesion stimulates Rac activity (Nakagawa et al., 2001; Noren et al., 2001). We recently reported that E-cadherin also stimulates the activation of Rac in MCF10A cells, and we linked this effect to E-cadherin stimulation of Rac activity in MCF10A cells (Liu et al., 2006). Since the results in Fig. 3 show that E-cadherin stimulates cyclin D1 mRNA levels independent of changes in ERK activity, we asked if the stimulatory effect of E-cadherin on cyclin D1 mRNA levels was dependent on Rac. Indeed, we found that expression of a dominant-negative construct, N17-Rac, inhibited cyclin D1 mRNA expression in a dose-dependent manner (Fig. 5A). As an independent approach to inhibiting Rac signaling, we infected MCF10A cells with an adenovirus encoding β2-chimerin, a Rac-specific GTPase activating protein (Caloca et al., 2003; Yang et al., 2005). β2-chimerin inhibited cyclin D1 mRNA expression in a dose-dependent manner (Fig. 5B) that was similar to that of the N17-Rac adenovirus.

Moreover, we found that inhibition of Rac signaling effectively blocked the induction of cyclin D1 mRNA after mitogen stimulation of suspended MCF10A cells (where E-cadherin is regulating cyclin

Fig. 4. Cyclin D1 half-life is not regulated by E-cadherin. MCF10A cells were transfected with control or E-cadherin siRNA during serum and growth factor starvation. (A) The starved cells were trypsinized, reseeded in suspension on agarose-coated dishes and stimulated with 10% FBS and growth factor cocktail for 6 hours to induce cyclin D1. Cycloheximide (CHX) was then added, and the cells were collected every 15 minutes for 1 hour. Collected cells were analyzed by western blotting using anti-cyclin D1 and anti-GAPDH (loading control). The cyclin D1 blot was exposed for 10 and 30 seconds (s) so that all bands could be readily detected and easily compared between samples (e.g. compare 10 s ‘control siRNA’ with 30 s ‘E-cadherin siRNA’). (B) MCF10A cells were transfected with control or E-cadherin siRNA and then infected with β-galactosidase (LacZ) (20 MOI) or cyclin D1 adenoviruses (2.5-20 MOI) during serum and growth factor starvation. The cells were trypsinized, reseeded in monolayer [M; β-galactosidase control only] or suspension (Susp) on dishes coated with collagen or agarose, respectively. The cells were stimulated with 10% FBS and growth factor cocktail for 9 hours. Cell lysates were analyzed by western blotting for cyclin D1, E-cadherin, and actin (loading control).

Fig. 5. Cell-cell- and cell-substratum-dependent expression of cyclin D1 mRNA is mediated by Rac. MCF10A cells were infected with adenoviruses encoding GFP, β-galactosidase (LacZ), N17Rac or β2-chimerin and serum starved. Control viruses were used at the highest MOI of the test virus. The infected, serum-starved cells were plated on collagen-coated dishes and stimulated with 10% FBS and growth factor cocktail. (A) Cells were mitogen stimulated for 9 hours, and the effect of N17-Rac on cyclin D1 mRNA was determined by QPCR. (B) Cells were mitogen stimulated for 9 hours, and the effect of β2-chimerin on cyclin D1 mRNA was determined by QPCR. (C) MCF10A cells were transfected with control or E-cadherin siRNA and then infected with β-galactosidase (LacZ) or N17-Rac adenovirus during serum and growth factor-starvation. Quiescent, transfected/infected cells were trypsinized, reseeded in monolayer (Mono) or suspension (Susp) on collagen- or agarose-coated dishes, respectively. The cells were stimulated with 10% FBS and growth factor cocktail for 12 hours. Total RNA was isolated and analyzed for cyclin D1 mRNA by QPCR.
that ERK activity has some role in the integrin-dependent induction of cyclin D1 mRNA in MCF10A cells because there is a partial inhibition of cyclin D1 mRNA and protein expression when integrin-dependent ERK activity is blocked by incubating MCF10A cells in suspension (refer to Fig. 1).

**E-cadherin-dependent expression of cyclin D1 contributes to efficient S-phase entry**

Our previous studies documented the proliferative effect of E-cadherin-dependent adhesions by plating cells at different densities (Liu et al., 2006). MCF10A cells plated at low density attached and spread on the substrate but the large majority of cells fail to form cell-cell adhesions. By contrast, plating cells at an intermediate density allowed for attachment, spreading, and efficient formation of cell-cell adhesions (Liu et al., 2006). These cell-cell adhesions were mediated by E-cadherin and associated with increased S-phase entry, typically ~1.5-3 fold. We exploited this system as a complementary approach to studying the link between E-cadherin, cyclin D1, and S-phase entry. We found that the increased incorporation of BrdU seen in MCF10A cells plated at a density that allowed for the formation of cell-cell adhesions (Fig. 7A, control siRNA) was associated with an increased expression of cyclin D1 mRNA (Fig. 7B, control siRNA). Knock-down of E-cadherin attenuated the contact-dependent increases in both BrdU incorporation and cyclin D1 gene expression (Fig. 7A,B, E-cad siRNAs). Conversely, ectopic expression of cyclin D1 released cells from their requirement for cell-cell contact in order to synthesize DNA (Fig. 7C). Taken together, our combined data show that the pro-mitogenic effect of E-cadherin in MCF10A cells can be explained by a Rac-dependent induction of cyclin D1 mRNA.

We consistently noticed a relatively small inhibitory effect of the E-cadherin siRNAs on cyclin D1 gene expression in the absence of cell-cell adhesion (Fig. 7B). Although we cannot exclude the possibility of small off-target effects of these two siRNAs, a likely explanation is that these siRNAs are targeting the small percentage of MCF10A cells that form cell-cell adhesions even at low seeding densities.

**Discussion**

Our findings characterize the cell cycle regulatory mechanism by which E-cadherin promotes S-phase entry in MCF10A cells. By experimentally isolating the individual effects of cell-substratum and cell-cell adhesion, we show that E-cadherin-mediated cell-cell adhesion is sufficient to cooperate with growth factor signaling and stimulate cyclin D1 gene expression. These results reveal a striking difference between epithelial and fibroblastic cells in their requirements for cyclin D1 induction. Although both cell types require growth factor and adhesion receptor signaling to induce cyclin D1, fibroblasts depend on integrin-mediated adhesion whereas the adhesion requirement in these epithelial cells can be satisfied by signaling through either integrins or E-cadherin. Thus, our studies identify a cell type in which integrin signaling is dispensable for cyclin D1 induction.
E-cadherin regulates cyclin D1 via Rac

Consistent with the redundant effects of integrins and E-cadherins on cyclin D1 expression in MCF10A cells, we find that both of these adhesion receptors use a Rac-dependent signaling pathway to induce cyclin D1 mRNA. We and others have previously shown that both ERK and Rac can induce cyclin D1 gene expression, but most of the studies with the Rac pathway have relied on overexpression of an activated Rac allele (Gjoerup et al., 1998; Joyce et al., 1999; Klein et al., 2007; Page et al., 1999; Westwick et al., 1997). We previously showed that endogenous Rac can induce cyclin D1 mRNA in fibroblasts, but this pathway is only seen upon deliberate inhibition of Rho (Welsh et al., 2001). By contrast, the results described here show that endogenous Rac signaling plays a major role in regulating cyclin D1 gene expression in MCF10A mammary epithelial cells. Neither E-cadherin nor Rac1 knock-down strongly affected ERK activity, consistent with our previous studies indicating that Rac can stimulate cyclin D1 gene expression independently of ERK (Welsh et al., 2001).

E-cadherin has long been thought of as a tumor- and growth-suppressor. Loss or downregulation of E-cadherin has been found in several types of cancer (Hajra and Fearon, 2002), and re-expression of functional E-cadherin reduced invasiveness of epithelial tumor cells and arrested tumor metastasis in late stage tumor progression in transgenic mice (Perl et al., 1998; Vleminkx et al., 1991). E-cadherin appears to play a role in contact inhibition of proliferation as cells reach confluence in cell-culture (Kandikonda et al., 1996; Takahashi and Suzuki, 1996).

Despite the evidence that E-cadherin suppresses proliferation, other studies support a proliferative role for E-cadherin. Consistent with our data, inhibition of E-cadherin-mediated adhesion with a dominant negative E-cadherin adenovirus inhibited proliferation in keratinocytes (Zhu and Watt, 1996). In contrast to most cancer types, more than 85% of ovarian carcinomas have elevated E-cadherin levels, and suppression of E-cadherin function decreases proliferation (Reddy et al., 2005). E-cadherin expression is also present in aggressive inflammatory breast cancer and some derivate metastases (Charafe-Jauffret et al., 2004; Kowalski et al., 2003). In the context of normal developing tissues, loss of E-cadherin leads to inhibition of proliferation in the blastocyst, the mammary gland and the hair follicle (Boussadia et al., 2002; Ohsugi et al., 1997; Tinkle et al., 2004). This link between cadherin engagement and proliferative capacity may exist to provide a growth advantage to epithelial cells appropriately positioned within their native tissues, and to support proliferation in early development when extracellular matrix is poorly organized. It would be interesting to know the degree to which Rac-dependent regulation of cyclin D1 underlies these diverse pro-proliferative effects of E-cadherin.

Materials and Methods

Cell culture

MCF10A cells were maintained in 1:1 low glucose Dulbecco’s modified Eagle’s medium (DMEM):Ham’s F12 nutrient medium supplemented with 10 mM Hepes buffer pH 7.4, 5% horse serum (Invitrogen), growth factor cocktail (20 ng/ml epithelial growth factor (EGF; BD Biosciences), 10 µg/ml insulin (Sigma), 0.5 µg/ml hydrocortisone (Sigma) and 100 ng/ml cholera toxin (List Biologicals) and 1% gentamycin (Invitrogen). For cell cycle time-course experiments, MCF10A cells were seeded at 2×10^6 cells per 100-mm dish or at 1×10^5 per 60-mm dish and allowed to attach and spread overnight in maintenance medium before synchronization by serum and growth factor starvation in 1:1 glucose DMEM-Ham’s F12 nutrient medium, 10 mM Hepes pH 7.4, with 1 mg/ml BSA and gentamicin for 2 days. Unless noted otherwise, the quiescent MCF10A cells were trypsinized and suspended in fresh starvation medium for 30 minutes at 37°C prior to reseeding onto 100-mm or 60-mm dishes coated with 1.27 µg/cm² collagen (monolayer) or 1% agarose (suspension). The cells were stimulated with 10% FBS and growth factor cocktail. For sample collection pre- or post-stimulation, cells were washed once with cold PBS, 1 mM NaVO₅, scraped, split into separate tubes for protein and mRNA analysis, collected by centrifugation, quick frozen, and stored at –80°C prior to analysis.

To assess cell aggregation in suspension, quiescent MCF10A cells (1×10⁶) were seeded in 150-mm agarose-coated dishes and stimulated in 20 ml 10% FBS (unless indicated) and growth factor cocktail in DMEM-F12. At selected times, a 1 ml aliquot of each suspension culture was seeded into a 35-mm dish containing coverslips coated with 25 µg/ml poly-L-lysine (Sigma). Cells were allowed to attach for 5 minutes. The coverslips were gently washed with cold PBS, fixed with 3.7% formaldehyde in PBS, and examined by phase-contrast microscopy or immunofluorescence microscopy using a 4× objective.

Epifluorescence and phase-contrast microscopy

Quiescent MCF10A were seeded in 100-mm dishes containing autoclaved glass coverslips coated with collagen and then stimulated with 10% FBS and growth factor cocktail in DMEM-F12. Cells were fixed in 3.7% formaldehyde, briefly incubated with 50 mM NH₄Cl in PBS, and then blocked and permeabilized simultaneously with 0.5% Triton X-100 in PBS with 0.2% BSA and 2% goat serum for at least 30 minutes.
siRNA transfection and adenosine infection

For RNA interference (RNAi) experiments, MCF10A cells were seeded in 35- or 100-mm culture dishes (0.5 × 10^6 cells/cm²) in maintenance medium without antibiotics the day before transfection. Cells were washed three times with serum- and growth-factor-free OptiMEM (Invitrogen) and then transfected with siRNA oligonucleotides (Ambion) for human E-cadherin (150 nM 5′-GAGUGAUAU-UUGAAGAGUGU-3′; ID44988 or 200 nM 5′-GACGUACAGCAGCUCU-AUU-3′; ID416381), human Rac1 (150 nM 5′-GAUAUACACCUCCAG-UCU-3′; ID45588), or irrelevant control (targeting mouse LIM kinase 1; 5′-GGUAAUGAGGGCGUAUCUG-3′; ID516310) in the presence of 10 μl Lipofectamine 2000 (Invitrogen) per well. The cultures were incubated in this serum-free medium for an additional 24 hours before use in experimentation. When adenosine infection was combined with RNAi, the cells were transfected with siRNA as described above, and the adenosine was added for 16 hours, beginning 24 hours after starvation. The adenosine was then removed and the medium was replaced with fresh serum-free medium for a total starvation time of 48 hours.

Analysis of cyclin D1 stability

Quiescent MCF10A cells that had been transfected with control or E-cadherin siRNA oligonucleotides were trypsinized, reseded (~3 × 10^5) in 100-mm dishes coated with agarose, and stimulated with 10% FBS and growth factors for 6 hours to allow for the expression of cyclin D1. Cyclin immunoblot samples were collected every 15 minutes for 1 hour and analyzed for cyclin D1 and GAPDH by western blotting.

We thank Jeffrey Albrecht (Hennepin County Medical Center, Minneapolis, MN), Marcelo Kazanietz (University of Pennsylvania) and Anne Ridley (University of London) for adenoviruses. This work was supported by NIH grants CA72639 to R.K.A., HL73305 to C.S. and CA078731 to V.M.W. A.K.F. was supported by pre-doctoral training grant 25-CA-101871. W.F.L. was supported by the National Training Grant.

References


